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KT Kavitha

Corresponding Author,
 Department of Veterinary
 Parasitology, Veterinary
 College and Research Institute,
 Orathanadu, Tamil Nadu
 Veterinary and Animal
 Sciences University,
 Tamil Nadu, India

A Latchumikanthan

Department of Veterinary
 Parasitology, Veterinary
 College and Research Institute,
 Orathanadu, Tamil Nadu,
 India

S Parthiban

Department of Biotechnology,
 Madras Veterinary College,
 Chennai, Tamil Nadu, India

R Nithyaselvi

Livestock Farm Complex,
 Veterinary College and
 Research Institute,
 Orathanadu, Tamil Nadu,
 India

M Palanivelrajan

Department of Veterinary
 Medicine, Veterinary College
 and Research Institute,
 Udumalpet, Tamil Nadu
 Veterinary and Animal
 Sciences University,
 Tamil Nadu, India

Corresponding Author:

KT Kavitha

Corresponding Author,
 Department of Veterinary
 Parasitology, Veterinary
 College and Research Institute,
 Orathanadu, Tamil Nadu
 Veterinary and Animal
 Sciences University,
 Tamil Nadu, India

Molecular detection of *Buxtonella sulcata* in Buffalo calves (*Bubalus bubalis*) from Orathanadu, Tamil Nadu

KT Kavitha, A Latchumikanthan, S Parthiban, R Nithyaselvi and M Palanivelrajan

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Abstract

Buxtonella sulcata is an intestinal ciliate commonly found in ruminants, yet their diagnosis is often complicated by morphological similarities with other ciliate genera. In the present study, *Buxtonella* cysts were detected in 44% (n=11) of fecal samples collected from buffalo calves in Orathanadu. Microscopically, the cysts observed were spherical, thick walled with kidney-shaped macronucleus and rounded micronucleus and measured 68-78 µm in length. The infection intensities ranged from 600 to 1000 cysts per gram of faeces. Due to the close morphological resemblance between *Buxtonella* and *Balantidium* cysts, molecular identification and confirmation were carried out by extracting DNA from morphologically identified *Buxtonella* spp. positive faecal samples, followed by specific PCR targeting the ITS1-5.8S rRNA-ITS2 region of *Buxtonella* spp. A 530 bp amplicon was obtained from all 11 positive samples. One representative amplicon was randomly subjected for Sanger sequencing, and contigs were assembled using BioEdit v7.0.9 software. Nucleotide sequence analyses revealed that the Orathanadu isolate shared 94.1-100% sequence similarity with previously published *Buxtonella* isolates from buffaloes and cattle. Phylogenetic analysis revealed that the Orathanadu isolate clustered closely with previously reported *B. sulcata* from buffaloes and cattle. This study provides molecular evidence for the presence of *B. sulcata* infection in buffalo calves and highlights the importance of molecular diagnostics for accurate species-level identification.

Keywords: Buffalo calves, *Buxtonella sulcata*, molecular identification, ITS1-5.8S-rRNA-ITS2 region, phylogeny

Introduction

Buxtonella species are intestinal ciliates belonging to the phylum Ciliophora, class Litostomatea, and family Balantidiidae, and are closely related to *Balantidium coli*, a parasite that infects humans and pigs [1]. These are large cyst-forming ciliates that infect ruminants and monkeys. Among domestic ruminants, *Buxtonella sulcata* is commonly reported in the large intestine of cattle and buffaloes [2]. These ciliates are generally regarded as commensal organisms inhabiting the caecum and colon; however, under conditions of stress, poor hygiene, or concurrent infections, they may become opportunistic pathogens leading to clinical enteritis, diarrhea, and weight loss [3, 4]. The life cycle of *B. sulcata* comprises two stages: the trophozoite and the cyst. The trophozoite is the motile, feeding, and replicative stage found in the large intestine, while the cyst is the resistant, infective stage excreted with feces. Infection occurs through the fecal-oral route by ingestion of food or water contaminated with cysts [5]. Once ingested, excystation occurs in the host intestine, releasing trophozoites that colonize the large bowel.

The prevalence of *Buxtonella* infection in buffaloes varies widely across regions and is influenced by climatic factors, husbandry practices, and the health status of animals. Studies from tropical and subtropical regions, including India, have reported prevalence rates ranging from 5% to 40% in cattle and buffaloes [2, 6]. Although infections are often subclinical, *Buxtonella* has been associated with gastrointestinal disturbances and diarrhea, particularly in young or debilitated animals [7]. The severity of infection tends to increase in immunocompromised animals, resulting in chronic health complications, reduced production performance, and mortality in severe cases [8].

Despite being reported for decades, *Buxtonella* infections in buffaloes remain under diagnosed due to their morphological resemblance to other intestinal protozoa, such as *B. coli*, and the lack of molecular diagnostic tools in routine veterinary parasitology [1]. So far, *B. sulcata* infection is not reported in humans. Given the economic importance of buffaloes as dairy and draught animals in many developing countries, understanding the epidemiology, pathogenic potential and control of *Buxtonella* infection is crucial for maintaining animal health and productivity.

Materials and Methods

Sample collection: A total of 25 faecal samples were collected from buffalo calves aged 2 months to 1 year during June 2025, all of which had a history of diarrhoea, at the Livestock Farm Complex, Veterinary College and Research Institute, Orathanadu, Tamil Nadu, southern India.

Faecal examination

The faecal samples were processed by direct wet mount, sedimentation and flotation technique by standard protocols [3]. The samples were examined under microscopy and the parasite stages were identified based on their morphological characters [4]. The intensity of infection in positive faecal sample was measured using the McMaster egg counting method [9].

Genomic DNA Isolation

Faecal samples positive for *Buxtonella* cysts were fixed in 70% ethanol. Total genomic DNA was extracted using the QIAamp DNA Stool Mini Kit (Qiagen, Germany) according to the manufacturer's instructions. Prior to extraction, cyst-positive faecal samples were resuspended in 400 μ L of lysis buffer (100 mM Tris-HCl [pH 8.0], 25 mM EDTA, 10% SDS), subjected to freeze-thaw cycles by freezing in liquid nitrogen for 3 min and thawing at 37 °C for 3 min. DNA concentration and purity were assessed by spectrophotometry (Nanodrop), and the extracted DNA was stored at -20 °C.

PCR amplification

PCR was performed on all 11 morphologically positive samples to amplify the internal transcribed spacer (ITS1-5.8S rRNA-ITS2) region of *Buxtonella* species using the published forward primer B5D (5'-GCT CCT ACC GAT ACC GGG T-3') and reverse primer B5R (5'-ATA TGC TTA AGT TCA GCG GG-3') as described by Ponce-Gordo *et al.* [10]. The reaction was carried out in a total volume of 20 μ L, comprising 6 μ L of nuclease-free water, 10 μ L of Ampliqon Master Mix, 1 μ L each of forward and reverse primers (20 pmol/ μ L), and 2 μ L of template DNA. PCR amplification was performed under the following thermal cycling conditions: initial denaturation at 94 °C for 10 min; 30 cycles of denaturation at 94 °C for 1 min, annealing at 60 °C for 1 min, and extension at 72 °C for 1 min; followed by a final extension at 72 °C for 5 min. Amplified products were confirmed by electrophoresis on a 1.5% agarose gel using a submarine horizontal electrophoresis unit (Bio-Rad, USA).

Sequence analysis

One PCR-positive amplicon was randomly selected and subjected to Sanger sequencing at Eurofins Genomics Pvt. Ltd. (Bengaluru, India) to confirm the identity of *Buxtonella* species. The obtained nucleotide sequences were analyzed using BLAST and Gene Tool software. Sequences generated in the present study were aligned with reference *Buxtonella* sequences retrieved from the GenBank database using ClustalX v2.1. Phylogenetic analysis was performed using the Maximum Likelihood method with 1,000 bootstrap replicates in MEGA v7.0.7 to confirm species identity.

Results

In the present study, faecal examination revealed that 44% (n=11) of the faecal samples from buffalo calves were positive for *Buxtonella* cysts. Microscopically, the cysts observed were spherical, thick-walled with a kidney-shaped macronucleus and a rounded micronucleus, and measured 68-78 μ m in length (Fig. 1). The cysts were morphologically similar to those of *Balantidium* species. The infection intensities in the positive faecal samples ranged from 600 to 1000 cysts per gram of faeces. The marked morphological similarity of cysts between the genera *Buxtonella* and *Balantidium* necessitated the use of molecular tools for accurate species identification.



Fig 1: *Buxtonella* cyst has spherical, thick-walled with a kidney-shaped macronucleus and a rounded micronucleus at 40X

The ITS1-5.8S rRNA-ITS2 region of *Buxtonella* was successfully amplified in all 11 samples that were morphologically identified as *Buxtonella* species yielding an amplicon of approximately 530 bp, as visualized on a 1.5% agarose gel (Fig. 2). One randomly selected amplified product from the Orathanadu isolate was sequenced, and its phylogenetic relationship with previously published isolates was analyzed. BLAST analysis revealed that the ITS1-5.8S rRNA-ITS2 sequence of the Orathanadu isolate shared 94.1-100% nucleotide identity with isolates reported from buffaloes and cattle. Phylogenetic analysis using the Maximum Likelihood (ML) method demonstrated that the Orathanadu isolate clustered with published *B. sulcata* sequences with bootstrap support exceeding 90% (Fig. 3), thereby confirming molecular positivity.

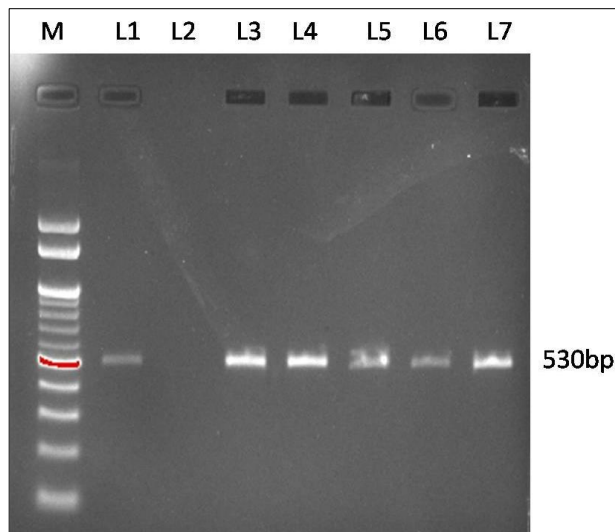


Fig 2: PCR Amplification of the ITS1-5.8S rRNA-ITS2 Region of *Buxtonella sulcata*

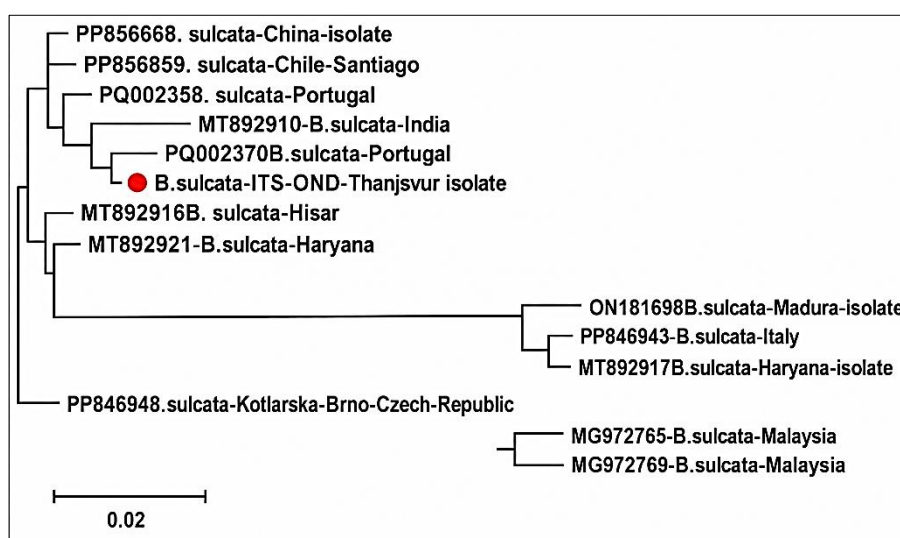


Fig 3: Phylogenetic analysis of ITS1-5.8S rRNA-ITS2 sequences from *Buxtonella sulcata* using Maximum Likelihood (ML) Method with Tamura-Nei model with 1000 bootstraps

Lane M- 100 bp ladder; Lane 1- known positive *B. sulcata* DNA; Lane 2- Non-template control, Lane 3 to 7 - field samples. The specific PCR product 530 bp is labeled separately.

Discussion

The present study confirmed the occurrence of *Buxtonella sulcata* infection in buffalo calves based on both morphological and molecular evidence. Microscopically, the cysts observed were spherical, thick-walled, and measured 68-78 μm , which closely corresponded with earlier descriptions of *B. sulcata* cysts reported in cattle and buffaloes [11]. However, reliance on cyst morphology alone had limited diagnostic value due to its strong resemblance to *B. coli*, frequently resulting in misidentification during routine fecal examinations [9, 12]. Consequently, molecular techniques provided more reliable diagnostic tools, enabling accurate differentiation between these two ciliates and ensuring species-level confirmation [13]. In India, several studies had documented the morphological identification and prevalence of *B. sulcata* infection in cattle and buffaloes [14, 15]. Notably, Kalkal and Sangwan [15] reported an overall prevalence of 54.5% in buffaloes from the Hissar district of Haryana, while Maharana *et al.* [14] recorded prevalence

rates of 4.57% and 15.79% in cattle and buffaloes, respectively, from Gujarat. Kumar *et al.* [8] reported an incidence of 35% from buffaloes of Gujarat. The variations in the percentages of infection could be due to many factors, such as environmental conditions, animal, farm management practices and stress factors.

In the present study, the infection intensity observed in faecal samples (600-1000 cysts/g) reflected a substantial cyst burden in calves. High cyst output had been associated with diarrheic conditions and poor nutritional status in young ruminants, although clinical manifestations varied depending on host immunity and the presence of co-existing enteric pathogens [16]. A previous study reported that animals exhibiting diarrheal symptoms had significantly higher infection rates than those with normal feces [12]. In animals with *B. sulcata* infection intensities exceeding 500 cysts per gram of feces, the frequency of diarrhea distinctly increased, and when intensities exceeded 2000 cysts per gram of feces, diarrhea frequency reached 100% [9]. *B. sulcata* has been recognized as an opportunistic pathogen in ruminants and its infections were reported to be highly prevalent among cattle and buffaloes worldwide [17].

Molecular characterization using the ITS1-5.8S rRNA-ITS2 region proved critical for species identification. The

amplicon size (~530 bp) obtained in this study corresponded well with previous reports on *Buxtonella* ITS sequences from cattle and buffaloes [13, 18]. ITS regions were known for their hypervariability, offering higher discriminatory power than conserved ribosomal genes and enabling clear differentiation among ciliate species [5]. The high sequence similarity (94.1-100%) between the Orathanadu isolates and published *Buxtonella* sequences supported the classification of these isolates within the *Buxtonella* genus. Phylogenetic analysis further demonstrated that the isolates clustered with *Buxtonella sulcata* from buffaloes and cattle, in agreement with earlier findings that ruminant *Buxtonella* species share a close evolutionary relationship despite host differences [1, 18]. This close genetic relatedness suggests either cross-species transmission or shared ancestral lineages within domestic ruminants.

These findings contribute to the existing knowledge on protozoan infections in buffaloes and emphasize the importance of integrating molecular diagnostics into routine parasitological examinations and differential diagnosis. Accurate species-level molecular identification is particularly critical in regions where buffaloes are of significant economic importance and where mixed infections with other enteric pathogens are common. Continued surveillance and molecular characterization will help elucidate the epidemiology, host specificity and pathogenic potential of *Buxtonella* spp. in livestock populations. Such molecular data not only enhance diagnostic accuracy but also provide valuable insights into host specificity, geographical distribution, and the potential zoonotic importance of the parasite.

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